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Heme oxygenase-1 contributes to the protective effect of resveratrol against endothelial dysfunction in STZ-induced diabetes in rats

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ABSTRACT

Endothelial dysfunction is a common complication of diabetes that mainly stems from increased reactive oxygen species, which makes antioxidants of great benefit. Resveratrol (RSV) is an antioxidant that shows protective effects in a variety of disease models where the ameliorative effect appears to be mediated, in part, via heme oxygenase-1 (HO-1) induction. However, the pathophysiological relevance of HO-1 in the ameliorative response of RSV in endothelial dysfunction is not clearly defined. The present study was conducted to investigate whether HO-1 plays a role in diabetes-induced vascular dysfunction. Streptozotocin-diabetic rats were treated with RSV (10 mg/kg) in presence or absence of an HO-1 blocker, Zinc protoporphyrin (ZnPP) to assess vascular function and indicators of disease status. We found that RSV treatment significantly abrogated diabetes induced vascular dysfunction. This improvement was associated with the ability of RSV to decrease oxidative stress markers alongside a reduction in the aortic TGF-β expression, elevation of NOS3 expression and aortic nitrite concentration as well as HO activity. These ameliorative effects were diminished when ZnPP was administered prior to RSV. Our results clearly demonstrate the protective effects of RSV in diabetes-associated endothelial dysfunction and verified a causal role of HO-1 in this setting.

1. Introduction

Diabetes mellitus is one of the most prevalent diseases worldwide with devastating complications. The state of hyperglycemia that characterizes diabetes results in both microvascular and macrovascular complications. Loss of the modulatory role of the endothelium is a critical factor in development of diabetic vascular disease. The molecular mechanisms of diabetes induced endothelial dysfunction have been extensively studied. Many reports have clearly defined the role of reactive oxygen species (ROS) where increased oxidative stress was associated with activation of various metabolic pathways such as polyol pathway and hexosamine pathway in addition to activation of protein kinase C (PKC) and enhancement of formation of advanced glycation end products (AGEs) [1–4]. These changes are associated with increased vasoconstrictor peptides e.g. endothelin-I and angiotensin-II in addition to reduction in nitric oxide production leading to endothelial dysfunction [5–7]. Thus, antioxidants play a major role in counteracting diabetes-induced vascular impairment [8,9].

Resveratrol (RSV) is a polyphenol initially isolated from *Veratrum grandifolorum* and then extracted from other plants such as peanuts (*Arachis hypogaea*) and different types of berries (*Vaccinium angustifolium Aiton* and *Vaccinium ashei Reade*) [10,11]. Interestingly, in clinical trials, RSV improved cerebrovascular function in patients with type 2 diabetes mellitus [12], and increased insulin sensitivity [13]. In addition, RSV attenuated the endothelial dysfunction associated with glucose intolerance [14]. Various molecular targets are suggested to mediate this effect including inhibition of angiotensin II-induced endothelin-1 gene expression, activation of sirtuins and associated increase in expression and activity of NOS3 [15,16]. In addition, RSV induced the activity of telomerase resulting in increased number and activity of epithelial progenitor cells and decreased senescence [17]. Moreover, the antioxidant activity of RSV is also considered a potential contributor to the protective effect on endothelium [18].

Heme oxygenase (HO) is one of the antioxidant enzymes which mediate heme degradation into carbon monoxide, iron and biliverdin. Biliverdin is subsequently converted into bilirubin via biliverdin reductase [19]. The downstream products of HO induce cytoprotective effects. Thus, induction of HO produced beneficial effects in various disease models such as atherosclerosis [20] and organ transplantation [21]. Additionally, In our previous work, we demonstrated the salutary effect of HO on diabetic myocardium [22]. However, there is a controversy in the regulatory role of HO-1 in endothelial dysfunction. One

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study showed the beneficial effect of HO-1 induction on endothelial function in Zucker rats [23]. Though, another study demonstrated that upregulation of HO-1 in mice increased blood pressure via inhibition of endothelial dependent relaxation and did not improve angiotensin II induced hypertension in mice [24,25]. Although RSV stimulates HO-1, little information is available about the role of HO-1 in the ameliorative effect of RSV on endothelial dysfunction associated with diabetes.

This study was conducted to investigate the role of HO-1 in mediating vasoprotective effect of RSV against diabetes-induced endothelial dysfunction. To test this hypothesis, we examined the effect of RSV when administered alone or in combination with Zn protoporphyrin (ZnPP), an HO-1 inhibitor, on endothelial dysfunction in STZ-diabetic rats.

2. Methods

2.1. Chemicals

Resveratrol (RSV) was purchased from Nutravit, USA. While, Zinc protoporphyrin IX (ZnPP) was obtained from Santa Cruz, USA. Streptozotocin (STZ), phenylephrine (PE), acetylcholine (ACh), and sodium nitroprusside (SNP) were purchased from Sigma Aldrich, USA. Primary antibodies for detection of NOS3 (Catalog No.PA1–037), TGF-β1 (Catalog No.MA5–15065) as well as HRP-conjugated secondary antibody (Catalog No. NBP2-30348H) were purchased from Thermo Fisher Scientific. Radioimmunoprecipitation assay (RIPA) lysis buffer was provided by Bio BASIC INC. (Marhham Ontario L3R 8 T4 Canada).

2.2. Animals

Adult male Wistar rats (200–220 g) were purchased from the Experimental Animal Center of Nahda University (Beni Sueif, Egypt). Rats were subjected to acclimatization period of one week and were kept under constant environmental conditions throughout the experiment. Animals were exposed to a 12:12 h dark: light cycle and were provided with standard rat chow diet (El-Nasr Company, Abou Zaabal, Cairo, Egypt). Since RSV was provided in drinking water, rats were single-housed and drinking water was provided using non-spill bottles.

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [26]. The study protocol was approved by members of The Research Ethics Committee at the Pharmacology &Toxicology Department, Faculty of Pharmacy, Minia University, Egypt (approval number 023/18).

2.3. Induction of Type-1 diabetes

Overnight fasted rats were intraperitoneally injected with STZ in a dose of 50 mg/kg. STZ was dissolved in cold saline (4 °C) immediately before injection while the control groups were injected with vehicle [27]. A 10% glucose solution was provided for the first 24 h after injection to counteract initial hypoglycemia induced by the release of insulin via the action of STZ on pancreatic cells. Blood glucose was measured on day 3 through tail tipping using a commercial glucometer (Precicheck, FIA Biomed, Germany). Rats having blood glucose levels > 250 mg/dl were considered diabetic and included into the study.

2.4. Experimental design

Rats were randomly assigned to 6 groups; 1- Control group (non diabetic rats treated with vehicle), 2- Control + RSV group (non diabetic rats treated with RSV: 10 mg/kg/day [28] for 4 weeks in drinking water), 3- Diabetic group (diabetic rats treated with vehicle) 4- Diabetic + RSV group (diabetic rats treated with RSV), 5- Diabetic + ZnPP +RSV group (diabetic rats treated with ZnPP:10 μmole/kg/week [29]; I·P for 4 weeks + RSV) and finally 6- Diabetic + ZnPP group (diabetic rats treated with ZnPP).

Resveratrol in a dose of 2.5–5 mg/kg has been shown in previous studies to improve hyperglycemia associated with diabetes [30,31]. However, using these doses to test its direct effect on vascular function cannot be conclusive as any improvement can be attributed to the initial lowering of blood glucose level. Interestingly, Schmatz, Schetinger [32] showed that RSV in a dose of 10 mg/kg did not show an effect on blood glucose level in STZ-diabetic rats [33]. Thus, we chose to work using this dose to test the direct effect of RSV on vascular function without an effect on glycemic control.

2.5. Tissue collection

At the end of the experimental period, rats were anaesthetized using pentobarbital (50 mg/kg [34]) and blood samples were collected by exsanguination for separation of sera. Aortae were rapidly isolated and prepared for functional assessment and biochemical analysis. For functional assessment, aortae were rapidly immersed in cold Krebs-Henseleit buffer (4 °C) and the perivascular adipose tissue was removed under a dissecting microscope. For biochemical analyses, aortae were flash frozen in liquid nitrogen and stored at −20 °c until the time of homogenization. To overcome elasticity of aortic tissue which limits the efficiency of homogenization, aortae were immediately immersed into liquid nitrogen so that the tissue becomes brittle then immediately homogenized using a motor driven homogenizer (LabGEN7, Cole-Parmer, USA) in nine volumes of phosphate buffered saline (4 °C) for assessment of oxidative stress parameters or in RIPA buffer (1 ml lysis buffer/100 mg tissue) for western blotting. Homogenates were centrifuged at 15000*g* for 10 min at 4 °C. The supernatant was withdrawn and protein content was determined using Biuret method [35] .

2.6. Assessment of vascular function

Aortae were immersed in Krebs-Henseleit buffer with the following composition in mmol/L; NaCl 118, KCl 4.6, $NaHCO_3$ 27.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.75, and glucose 11.1 (pH 7.4). Aortae were cut into 3–4 mm sized vessel rings and passed through a triangular stainless steel wire with caution to avoid any endothelial damage. Then, the rings were mounted in 10 ml Krebs-Henseleit buffer solution in organ baths and attached to routinely-calibrated isometric force displacement transducers (MLT0201/D, AD Instruments) connected to a Powerlab/8S data acquisition system and LabChart® software to measure and record changes in circumferential tension as units of milligram (mg). Tension was gradually adjusted to 1500 mg. The vessel was allowed to equilibrate over a period of 45 min during which, the solution was routinely replaced every 15 min. Vessel viability was assessed via measurement of tissue response to 80 mM KCl. A ring producing a contraction of 300 mg or greater, was considered viable. In addition, the endothelium was considered intact when ACh (1 μM) produces at least 60% relaxation in a 1 μM phenylephrine (PE) pre-contracted ring. Response to acetylcholine (1×10^{-9}-1×10^{-5} M) was recorded and % relaxation was calculated. Endothelium independent relaxation was assessed via detecting relaxation of PE pre-contracted rings in response to sodium nitroprusside (3×10^{-10}-1×10^{-7} M). Moreover, the change in contractile response to PE was examined in concentration range of (1×10^{-9}-1×10^{-5} M) and contraction was calculated as percentage of KCl induced contraction.

2.7. Determination of serum lipid peroxidation

Measurement of thiobarbituric acid reactive species (TBARS) as a marker of lipid peroxidation was performed using a commercial kit (Biodiagnostic, Egypt). The method depends on interaction of thiobarbituric acid with malondialdhyde (MDA) in acidic medium at temperature of 95 °C for 30 min. The reaction results in formation of pink color adduct. The color intensity is measured spectrophotometrically at 534 nm [36].

2.8. Determination of the catalytic activity of aortic superoxide dismutase (SOD)

Measurement of the catalytic activity of SOD was performed using an SOD kit, (Biodiagnostic, Egypt) according to the method suggested by which depends on reduction of nitroblue tetrazolium dye by phenazine methosulphate in presence of superoxide anion. Since SOD catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide, it inhibits the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye [37].

2.9. Determination of aortic heme oxygenase activity

The activity of HO in the aortic tissue was determined as previously described by *Abraham* et al, in which bilirubin, the end product of heme degradation, was extracted with chloroform. Bilirubin was determined spectrophotometrically with a scanning spectrophotometer and was defined as the difference between the absorbance at 463 and 520 nm. Bilirubin concentrations were read using a standard bilirubin curve. HO activity was expressed as pmol bilirubin/mg protein/h. [38].

2.10. Determination of nitrite concentration in aortic tissues

Measurement of nitrite concentration was performed using a commercial kit, (Biodiagnostic, Egypt). The method depends on interaction of nitrite with sulphanilamide (10 mM) and N–(1–naphthyl)-ethylenediamine (1 mM) resulting in formation of a deep purple azo compound. The absorbance of each sample was measured against blank at 540 nm. The concentration of nitrite was determined using a standard curve (3, 5, 10, 20, 30 nmol/ml) [39].

2.11. Western blot analysis for the detection of NOS3 and TGF-β1 protein expression

Protein concentration of 20 μg of each sample was mixed with an equal volume of 2× Laemmli sample buffer 4% sodium dodecyl sulphate, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M TrisHCl, pH 6.8). Each of the previous mixtures were boiled at 95 °C for 5 min to ensure denaturation of protein before loading on polyacrylamide gel. Samples were loaded on gel and a molecular weight marker (BLUelf prestained protein ladder, GeneDireX, Taiwan, Cat No.PM008–0500) was used to enable the determination of protein size and also to monitor the progress of an electrophoresis run. Protein separation occurred using TGX Stain-Free™ FastCast™ Acrylamide Kit (SDS-PAGE) obtained from Bio-Rad Laboratories(TNC, USA Catalog. No. 161–0181) in running buffer (25 mM Tris base, I90 mM glycine, 0.1% SDS, pH 8.3). The gel was run for 20 min at 50 V to allow migration of samples in stacking layer. The voltage was increased to 150 V to allow protein migration and separation in resolving layer to finish the run in about 1 h. Separation of proteins was visualized using stain-free technology and ChemiDoc TM imager. Membrane blotting was run for 7 min at 25 V to allow transfer of protein bands from gel to membrane using Bio-Rad Trans-BlotTurbo in a running buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3). Then, the membrane was blocked in blocking buffer, composed of tris-buffered saline with Tween 20 (TBST) (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) and 3% bovine serum albumin, at room temperature for 1 h.

For detection of protein bands of NOS3 and TGF-β1, membrane was incubated with a specific primary antibody after dilution of 1:1000 using (TBST). Incubation was done overnight at 4 °C. Then, membrane was washed three times, 10 min each with TBST at room temperature. For detection of bands, membrane was incubated with HRP-conjugated secondary antibody after dilution of 1:1000 in blocking solution for 1 h at room temperature and washed again three times, 10 min each with TBST at room temperature.

The chemiluminescent signals from protein bands were captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins against control sample after normalization by beta actin on the Chemi Doc MP imager. Data were expressed as fold change of control.

2.12. Statistical analyses

Results are expressed as mean ± standard error of the mean (SEM) and were analyzed for statistically significant differences using one way analysis of variance (ANOVA) followed by the Tukey–Kramer post analysis test to compare all groups at $p < .01$. Graph Pad Prism® was used for statistical calculation (Version 7.00 Windows, GraphPad Software, San Diego California USA).

3. Results

3.1. General parameters

Induction of diabetes resulted in a significant increase ($p < .0.1$) in fasting blood glucose level (203.0 mg/dl ± 10.4) as well as a significant reduction ($p < .01$) in body weight of animals (216.0 g ± 17.7) compared to control rats which had blood glucose level of 78.0 mg/dl ± 5.0 and body weight of 308.0 g ± 17.0. Treatment with RSV did not produce any significant change in blood glucose level or body weight. (data not shown).

3.2. Effect on vascular function

In aortic rings from control rats, acetylcholine (1×10^{-9} to 1×10^{-5} M) caused a concentration-dependent relaxation with maximal relaxation of 89.7 ± 5.0% at 10 μM. This relaxation was significantly ($p < .01$) impaired in aortic rings from diabetic rats which had maximal relaxation of 56.2 ± 9.8% as illustrated in Fig. 1(a). Aortae from diabetic rats treated with RSV for 4 weeks showed a significant improvement ($p < .01$) in acetylcholine-induced relaxation (86.67 ± 1.074%) as shown in Fig. 1(b). Interestingly diabetic rats treated with RSV showed no significant difference in their response to ACh when compared to control rats. Administration of ZnPP in combination with RSV to diabetic rats significantly mitigated the effects of RSV resulting in a maximal relaxation of 80.5 ± 2.2%. However, the relaxation was still significantly higher than diabetic control. Aortic rings from diabetic rats treated with ZnPP alone showed no statistical difference compared to diabetic control. Aortae from control rats treated with RSV showed an increase in the maximal relaxation compared to normal control (91.3 ± 5.0%), but the observed increase was statistically non-significant.

On the other hand, induction of diabetes produced no change in SNP induced relaxation nor did all other treatments as shown in Fig. 1(c). Similarly, none of the treatments produced any change in the contractile response to PE as shown in Fig. 1(d).

3.3. Effect on oxidative stress parameters

Induction of type I diabetes resulted in a significant elevation ($p < .01$) in serum TBARS as a marker of lipid peroxidation when compared to control rats. Daily administration of RSV (10 mg/kg) for 4 weeks produced a significant reduction ($p < .01$) in serum lipid peroxidation compared to diabetic rats as shown in Fig. 2(a). However, this effect was abolished upon using ZnPP in combination with resveratrol. RSV administered to control group did not show any significant reduction in lipid peroxidation when compared to control group.

Induction of diabetes was associated with a statistically significant increase in the activity of superoxide dismutase (SOD) as illustrated in Fig. 2(b). Treatment of diabetic rats with RSV for 4 weeks resulted in a

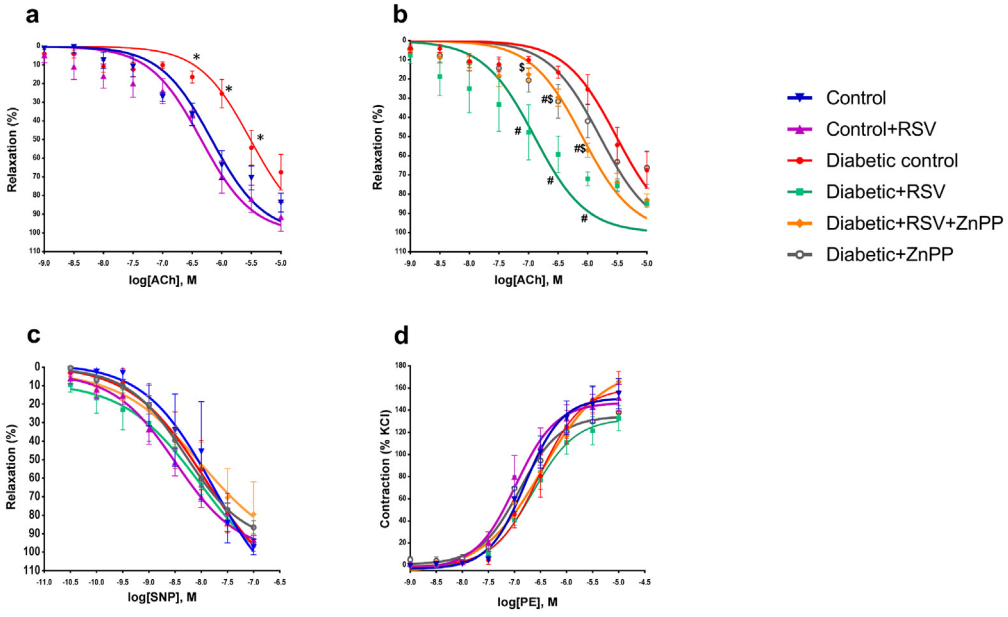

Fig. 1. Line graphs showing the effect of diabetes on vascular function and its alteration by various treatments. (a) and (b) represent acetylcholine dose response curve of various experimental groups. (c) Represents sodium nitroprusside dose response curve of various groups. (d) Represents phenylephrine dose response curve.
Data are presented as mean ± S.E.M. * Significantly different from control at $p < .01$. # Significantly different from diabetic control at $p < .01$. $ Significantly different from (Diabetic + RSV) at $p < .01$.
Control = rats treated with vehicle ($n = 6$), Control + RSV = control rats treated with resveratrol (n = 6), Diabetic control = diabetic rats treated with vehicle (n = 6), Diabetic + RSV = diabetic rats treated with resveratrol 10 mg/Kg/day (n = 6), Diabetic + ZnPP + RSV = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn protoporphyrin IX (10 μmole/kg/week) (n = 6) and Diabetic + ZnPP = diabetic rats treated with Zn protoporphyrin IX (10 μmole/kg/week) (n = 6).

further enhancement of the activity to a statistically significant level ($p < .01$). Blocking the action of RSV on HO-1 by using ZnPP attenuated the effect of RSV leading to a statistically significant decrease in SOD activity when compared to diabetic rats treated with RSV alone.

3.4. Effect on HO activity

Induction of diabetes significantly inhibited ($p < .01$) HO activity, while chronic treatment with RSV produced a significant elevation in the activity of HO in diabetic rats. Using ZnPP in combination with RSV abrogated the stimulatory effect of RSV on HO activity as shown in Fig. 3.

3.5. Effect on nitrite concentration

Induction of diabetes was associated with a significant decrease ($p < .01$) in aortic level of nitrite when compared to control as shown in Fig. 4(a). Treatment with RSV for 4 weeks produced a significant elevation in nitrite level. Using ZnPP, an HO-1 blocker in combination with RSV resulted in a significant abrogation of the effects of RSV in this regard. ZnPP administered alone to diabetic rats resulted in no significant change compared to diabetic control.

3.6. Effect on protein expression of NOS3

Induction of diabetes produced a significant ($p < .01$) reduction in expression of NOS3 as shown in Fig. 4(b & c). Chronic treatment with RSV (10 mg/kg) significantly enhanced NOS3 expression. Blockade of HO-1 activity by ZnPP significantly abrogated RSV effects in diabetic + ZnPP +RSV group. ZnPP when administered alone produced no significant change compared to diabetic control.

3.7. Effect on protein expression of TGF-β1

Induction of diabetes produced a significant (p < .01) elevation in the expression of TGF-β (p < .01) as illustrated in Fig. 5. Chronic treatment with RSV (10 mg/kg) significantly reduced the expression of TGF-β1. Blockade of HO-1 activity by ZnPP significantly abrogated RSV effects in this regard.

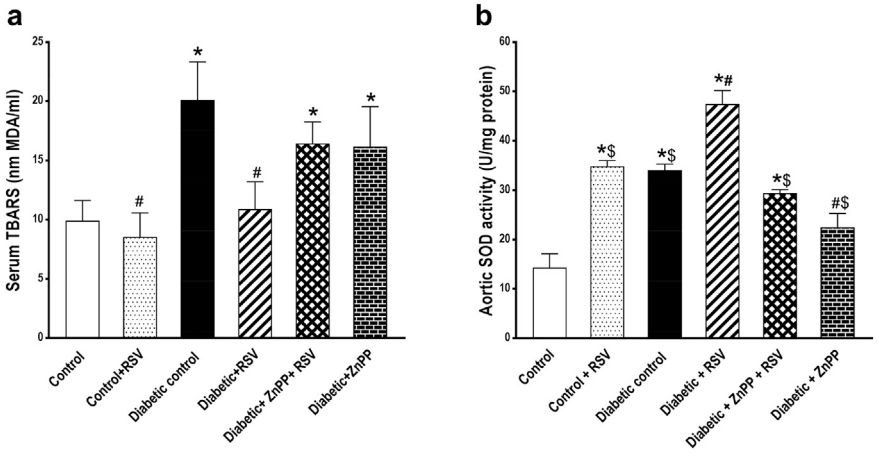

Fig. 2. Bar graphs showing the effect of diabetes on oxidative stress and its alteration by various treatments. (a) represents the change in the serum levels of TBARS of different experimental group and (b) shows the effect of diabetes on the activity of aortic superoxide dismutase as well as the effect of treatment with resveratrol and ZnPP. Data are represented as mean ± S.E.M. * Significantly different from control at $p < .01$. # Significantly different from diabetic control at $p < .01$. $ Significantly different from (Diabetic + RSV) at $p < .01$.
Control = rats treated with vehicle ($n = 5$), Control + RSV = control rats treated with resveratrol (n = 5), Diabetic control = diabetic rats treated with vehicle (n = 5), Diabetic + RSV = diabetic rats treated with resveratrol 10 mg/Kg/day (n = 5), Diabetic + ZnPP + RSV = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn protoporphyrin IX (10 μmole/kg/week) (n = 5) and Diabetic + ZnPP = diabetic rats treated with Zn protoporphyrin IX (10 μmole/kg/week) (n = 5).

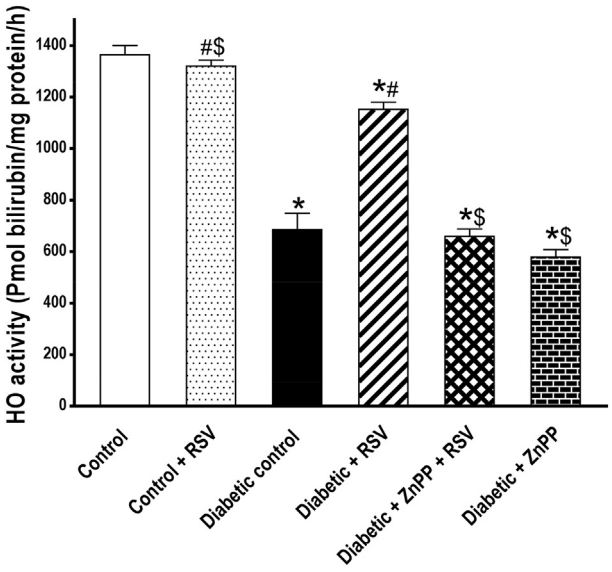

Fig. 3. A bar graph showing the change in aortic HO activity induced by diabetes and its alteration by various treatments.
Data are represented as mean ± S.E.M. * Significantly different from control at $p < .01$. # Significantly different from diabetic control at $p < .01$. $ Significantly different from (Diabetic + RSV) at $p < .01$.
Control = rats treated with vehicle ($n = 3$), Control + RSV = control rats treated with resveratrol (n = 3), Diabetic control = diabetic rats treated with vehicle (n = 3), Diabetic + RSV = diabetic rats treated with resveratrol 10 mg/Kg/day ($n = 3$), Diabetic + ZnPP + RSV = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn protoporphyrin IX (10 μmole/kg/week) ($n = 3$) and Diabetic + ZnPP = diabetic rats treated with Zn protoporphyrin IX (10 μmole/kg/week) ($n = 3$).

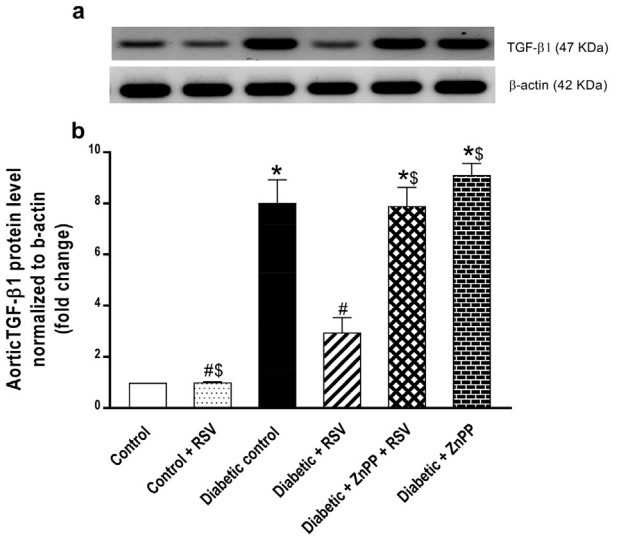

Fig. 5. Representative western blot bands showing the expression of TGF-β and β-actin (a) and a bar chart showing the quantitative analysis of TGF-β expression in diabetic state and its alteration by various treatments (b).
Data are presented as mean ± S.E.M. * Significantly different from control at $p < .01$. # Significantly different from diabetic control at $p < .01$. $ Significantly different from (Diabetic + RSV) at $p < .01$.
Control = rats treated with vehicle (n = 3), Control + RSV = control rats treated with resveratrol (n = 3), Diabetic control = diabetic rats treated with vehicle (n = 3), Diabetic + RSV = diabetic rats treated with resveratrol 10 mg/Kg/day (n = 3), Diabetic + ZnPP + RSV = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn protoporphyrin IX (10 μmole/kg/week) (n = 3) and Diabetic + ZnPP = diabetic rats treated with Zn protoporphyrin IX (10 μmole/kg/week) (n = 3).

4. Discussion

Improving endothelial function associated with diabetes is a reasonable approach to ameliorate cardiovascular complications, which represent a major cause of mortality in diabetic patients [40]. Activation of hemoxygenase enzyme was proposed as a protective pathway against endothelial dysfunction [41], however, the role of HO-1 induction shows some inconsistency in the literature. For example, a study by *Chen* et al (2004), on endothelial cells from human umbilical vein demonstrated a pro-oxidant effect of HO-1 in endothelial cells [42]. On the other hand, other studies demonstrated that HO-1 induction produced a protective effect by attenuation of endothelial cell senescence and apoptosis in human umbilical veins [43,44].

In our study, we evaluated the protective effects of resveratrol (RSV) on the vascular complications of diabetes induced by STZ in rats on a functional and a biochemical level. We investigated the role of HO-1 in mediating these effects. The study emphasized the ability of RSV to prevent the development of vascular complications and revealed that the protective effect of RSV can be partially attributed to its ability to enhance HO-1 activity. Our data also indicated that resveratrol and its associated overexpression of HO-1 reduced oxidative stress, which is a major cause of diabetes induced endothelial dysfunction. These results

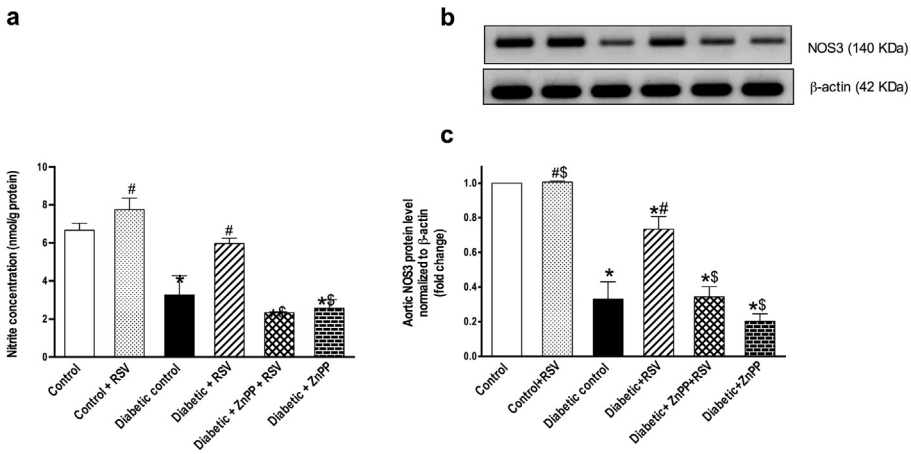

Fig. 4. (a) Bar graphs showing the change in concentration of aortic nitrite induced by diabetes and its alteration by various treatments. (b) Representatives western blot bands of the expression of NOS3 and β-actin and (c) shows the semi-quantitative analysis of NOS3 expression in diabetic state and its alteration by various treatments (c).
Data are represented as mean ± S.E.M. * Significantly different from control at $p < .01$. # Significantly different from diabetic control at $p < .01$. $ Significantly different from (Diabetic + RSV) at $p < .01$.
Control = rats treated with vehicle ($n = 3$), Control + RSV = control rats treated with resveratrol ($n = 3$), Diabetic control = diabetic rats treated with vehicle ($n = 3$), Diabetic + RSV = diabetic rats treated with resveratrol 10 mg/Kg/day ($n = 3$), Diabetic + ZnPP + RSV = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn protoporphyrin IX (10 μmole/kg/week) ($n = 3$) and Diabetic + ZnPP = diabetic rats treated with Zn protoporphyrin IX (10 μmole/kg/week) ($n = 3$).

highlight previous conclusions showing the ability of resveratrol to reduce oxidative stress and improve endothelial function [45,46]. The former study suggested that resveratrol plays its protective role by inhibiting TNF-α and NADPH oxidase while the later introduced adiponectin signaling as a target of RSV action.

In our study, RSV promoted the activity of HO-1, an effect previously explained by *Wang* et al (2017) by its ability to induce Nrf2 signaling [47]. Activation of Nrf2 signaling has been shown to exert protective effect in a mouse model of diabetic nephropathy [48] as well as in human microvascular endothelial cells [49]. The increased HO-1 activity leads to attenuation of diabetes-induced generation of ROS evident in a decrease in lipid peroxidation and an elevation of antioxidant defense mechanism through increased SOD activity in RSV treated diabetic rats. RSV failed to show this effect when combined with the HO-1 inhibitor, ZnPP emphasizing the role of HO-1 in this regard. Other reported mechanisms responsible for HO-1 mediated protection includes: increased NOS3 activity through the release of intracellular calcium and activation of PI3/AKT pathways [50], carbon monoxide mediated promotion of survival of endothelial cells [51,52] and bilirubin (a reduction product from biliverdin) induced antiapoptotic and antioxidant effects [53]. Interestingly, the ability of RSV and other HO-1 inducers is not evident in control (healthy) animals as we demonstrated in our data. The lack of effect on normal conditions was explained by the fact that Nrf2 (the molecular target of RSV) is not translocated into the nucleus unless a level of oxidative stress is achieved to generate ROS, which interacts with its cytoplasmic anchor and permits the translocation of Nrf2 and interaction with its DNA site of action [54].

Since the vascular complications of diabetes can be originally attributed to increased production of ROS [55], any pathway that counteract oxidative stress is expected to be beneficial. The beneficial effect of HO-1 on endothelial dysfunction was previously attributed to a direct anti-oxidative effect of HO-1 products and an indirect effect exerted by carbon monoxide through suppressing endothelial cells apoptosis [56]. However, we found that the improved endothelial function produced by RSV can be only partially related to its ability to induce HO-1 since this effect was not completely abrogated when combined with ZnPP. Thus, in our study we introduce HO-1 as one of the pathways mediating RSV vasoprotection where other previously reported mechanisms are probably involved. Such as its antioxidant effect, its ability to reduce TNF-a as well as its antiapoptotic effect [57,58].

The activity of the endothelial nitric oxide synthase (NOS3) has been long considered as a marker of endothelial function. Here, we reported that RSV induced NOS3 expression in the aortae of diabetic rats, which was associated with increased nitric oxide availability evident in an improved endothelial induced relaxation. Similar results were demonstrated by *Rodella* et al (2008) who also reported that RSV induced NOS3 expression and increased adiponectin levels leading to inhibition of vascular inflammation [46]. However, the study did not show the direct effect of HO-1 induction on the endothelial function. In our study, we demonstrated that the ability of RSV to induce NOS3 expression was attenuated when combined with ZnPP. A relation between HO-1 and NOS3 has been previously proposed for both to share a common pathway resulting from the induction of Nrf-2 signaling [59,60]. However, our data strongly suggests that NOS3 induction might be downstream to activation of HO-1. This postulation can be supported by a previous study showing the ability of carbon monoxide (a product of HO-1 activity) to induce NOS3 expression to exert its cardioprotective effect against ischemia reperfusion [61].

Transforming growth factor-β (TGF-β) is an important regulator of cell differentiation, proliferation and survival. TGF-β plays an important role in endothelium status depending on the balance between Smad3 and Smad5 signaling [62]. Thus, TGF-β signaling produces both proliferative and anti-proliferative effects depending on the cellular context. When TGF-β/Smad3 pathway is activated, it inhibits endothelial proliferation while activation of TGF-β/Smad5 pathway produces the opposite effect [63]. A direct relation between sustained hyperglycemia and Smad3 was reported [64] while inhibition of smad3 signaling lead to attenuation od endothelial function in a diabetic nephropathy model [65] In our study, induction of diabetes was associated with an eight-fold increase in aortic TGF-β protein expression, an effect which can be explained by a sustained hyperglycemia associated with our model.

Our data showed that chronic treatment with RSV reduced the expression of TGF-β to one half of the non-treated rats and this effect was abrogated when RSV was combined with ZnPP. The effect of HO-induction on TGF-β was previously reported in a study using quercetin as an HO-1 inducer where it suppressed TGF-β-induced collagen production in normal human lung fibroblasts [66]. A direct relation between Nrf2 signaling and TGF-β was reported in a study examining the role of Nrf2 in diabetic nephropathy [48]. The authors showed that Nrf2 activation leads to down regulation of TGF-β expression but the exact mechanism was not investigated. Our study suggests that HO-1 induction (which is downstream to Nrf2 signaling) might play a role in this pathway as inhibiting HO-1 by ZnPP resulted in a disappearance of its effect on TGF-β expression. Interestingly, activation of TGF-β has been shown to contribute to ROS generation by NADPH [67], which eventually leads to a reduction in NO bioavailability [68]. An effect, which supports our findings of reduced NOS3 expression and NO bioavailability in the diabetic aorta parallel to an increased TGF-β level. Examining the role of different signaling molecules to better understand the complex relation between TGF and HO-1 as well as performing specific staining of aortic tissue to see the impact of the enhanced TGF signaling al the tissue are limitations of our study which warrants further research.

5. Conclusion

Endothelial dysfunction plays a pivotal role in diabetic cardiovascular complication. Our data suggest that RSV ameliorates the endothelial dysfunction in STZ-induced diabetes through reduction of oxidative stress, improving aortic NOS3 expression and NO bioavailability as well as reducing TGF-β expression. We highlighted the importance of HO-1 signaling in RSV vasoprotective effect using ZnPP as an HO-1 inhibitor. Understanding the molecular mechanisms underlying the interaction between HO-1 and TGF-β signaling as well as their role in endothelial dysfunction will provide insight into the role of these factors in vascular homeostasis and may lead to the development of better strategies to circumvent diabetes induced vascular complications.

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Declaration of competing interest

The authors declare no conflict of interest.

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